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13. ABSTRACT (Maximum 200 Words) <u>Background:</u> Signaling through the epidermal growth factor receptor (EGFR) has been implicated in both effective wound healing and epithelial neoplasia. We have identified a novel function of the EGFR in support of epithelial cell survival, particularly in conditions of anchorage-independence. Furthermore, we have implicated MEK/MAPK signaling in this process. <u>Objective/hypothesis:</u> Define molecular mechanisms and pathways by which EGFR activation supports epithelial cell survival. Two specific aims focus on (1) posttranslational modification of relevant Bcl-2 family members by EGFR activation through MAPK-dependent mechanisms and, (2) STAT3 activation by deregulated EGFR signaling as observed in epithelial cancer. <u>Progress:</u> During the last funding period we have focused on modification of Bcl-2 family members during suspension culture. Specifically, we have characterized the pro-apoptotic Bcl-2 family member BIM to be upregulated during suspension culture and, potentially, phosphorylated by EGFR activation. These results are very encouraging and will be pursued further. The studies proposed under Specific Aim 2 have been completed and have been accepted for publication.				
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INTRODUCTION

The epidermal growth factor receptor (EGFR) is a type-1 receptor tyrosine kinase. Activation of the EGFR has been implicated in many aspects of cell biology pertaining to wound healing and malignant transformation of epithelial cells. Previous work from our laboratory underscored a novel function of the EGFR in support of cell survival of epidermal keratinocytes. Specifically, EGFR activation and signaling was observed to protect keratinocytes against induction of apoptosis through extracellular stressors (1-3). The major thrust of the research funded through **DAMD17-02-1-0216** is to further characterize the protective role of EGFR activation in situations in which keratinocytes encounter suboptimal extracellular matrix interaction, i.e. during wound healing and metastatic spread of malignant cells. We originally proposed to pursue two specific aspects of EGFR activation as they relate to cell survival. Specific Aim 1 focuses on posttranslational modifications of regulators of cell survival of the Bcl-2 family of molecules through EGFR activation and suspension culture. Specific Aim 2 deals with the question whether inappropriate signaling through the EGFR as observed in cancer cells leads to aberrant STAT3 activation, which in turn enhances cell survival.

BODY

This progress report will focus on accomplishments relating to Specific Aims 1 and 2 of the original proposal as we have made significant progress in both areas. A manuscript detailing results relating to Specific Aim 2 is currently in press.

Specific Aim 1

We have evaluated posttranslational modification of the proapoptotic Bcl-2 family member Bad during suspension culture and in the presence and absence of EGFR activation in HaCaT keratinocytes. In our last progress report, we have outlined difficulties in demonstrating Bad phosphorylation in HaCaT cells in forced suspension culture. After exhausting all technical options we conclude that such phosphorylation does not occur. However, based on a recent report (4) we turned our attention to posttranslational modification of a BH3-only proapoptotic Bcl-2 family member, BIM. We confirmed and extended earlier results in mammary epithelial cells that BIM expression is upregulated in the absence of matrix engagement. Furthermore, we identified, in Western blot analyses, a gelshift of BIM commensurate with posttranslational modification, particularly phosphorylation. Phosphorylation of Bcl-2 family members is a frequently observed regulatory mechanism to alter activity of these important apoptosis regulators. This gelshift occurred only in the presence of EGF in HaCaT cells in forced suspension. Thus, it may be related to MAPK activity (5, 6) which is maintained by EGFR activation in forced suspension culture. An attractive alternative is that JNK activity induced by placing cells in suspension culture may have a role to play in BIM expression or phosphorylation (7). These recent results are an exciting development in support of our original hypothesis and will be pursued with vigor during the next year.

Specific Aim 2

In Specific Aim 2 we hypothesized that STAT3 activation is a consequence of aberrant EGFR signaling as observed in epithelial cancer. This hypothesis was tested and the following results obtained:

- Consistent with reports in the literature (8-10) malignant epithelial tumor cells expressed increased levels of the EGFR relative to normal or immortalized keratinocytes.
- Similarly, EGFR phosphorylation in response to exogenous EGF was generally more pronounced in the tumor cells.
- STAT3 phosphorylation on Y705 was observed exclusively in malignant tumor cells and absent in all normal keratinocyte strains and in HaCaT cells.
- STAT3 Y705 phosphorylation was induced by EGF in tumor cells. However, in 3 of 5 cell lines tested STAT3 Y705 was observed even in the presence of EGFR inhibitors indicating that this phosphorylation event occurred independently of EGFR activation.

We now have completed the analysis of DNA binding activity of STAT3 in normal and malignant epithelial cells. This analysis confirmed that STAT3 activation under the culture conditions chosen here was restricted to tumor cells. Furthermore, we obtained additional evidence that EGFR-dependent MEK activation serves to negatively regulate STAT3 phosphorylation highlighting that EGFR activation affects STAT3 phosphorylation both, positively and negatively. Thus, instead of linear pathways to STAT3 activation by EGFR stimulation, more complex regulatory mechanisms appear to be operative. This work is in press in the AACR journal *Cancer Research* and due to be published in June/July 2004. In summary, the experiments proposed under this aim have been concluded allowing us to concentrate on the interesting results recently generated under Specific Aim 1 of the original proposal.

KEY RESEARCH ACCOMPLISHMENTS

- Identified upregulation of proapoptotic Bcl-2 family member BIM in keratinocyte suspension cultures
- Obtained tentative evidence for as yet unappreciated posttranslational modification of BIM in forced suspension cultures
- STAT3 phosphorylation levels are markedly different in normal and malignant keratinocytes. STAT3 Y705 phosphorylation is restricted to squamous carcinoma cells; paper describing these results in press.

REPORTABLE OUTCOMES

A manuscript describing the results under Specific Aim 2 is in press in Cancer Research; a preprint page proof copy is attached to this report.

CONCLUSIONS

During the first 24 months of funding we have accomplished the objectives laid out in Specific Aim 2 of the original proposal. In parallel, we have now obtained actionable results relating to Specific Aim 1 as outlined in the original proposal. Presently, these results point to a novel mechanism of EGFR-dependent regulation of BIM, a pro-apoptotic Bcl-2 family member. These results will be followed up and expanded upon during the current funding period.

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APPENDICES

Quadros, M.R.D., Peruzzi, F., Kari, C., and Rodeck, U. Complex Regulation of Signal Transducers and Activators of Transcription 3 activation in normal and malignant keratinocytes. *Cancer Research*. 64: (in press), 2004.

Complex Regulation of Signal Transducers and Activators of Transcription 3 Activation in Normal and Malignant Keratinocytes

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ABSTRACT

Previous work implicated activation of the signal transducer and activator of transcription (STAT)3 downstream of the epidermal growth factor receptor (EGFR) in the malignant phenotype of squamous carcinoma cells (SCCs). Here, we show that EGFR-dependent STAT3 activation is restricted to malignant keratinocytes. Specifically, constitutive and epidermal growth factor-induced phosphorylation of STAT3 on Y705 was observed only in SCC but not in either immortalized (HaCaT) or normal keratinocyte strains. Furthermore, STAT3 activation as determined by DNA binding assays was restricted to SCC and dependent on EGFR activation. Forced expression of EGFR in immortalized keratinocytes (HaCaT cells) was associated with enhanced EGFR activation but not STAT3-Y705 phosphorylation. EGFR-dependent activation of mitogen-activated protein kinase (MAPK) kinase 1 negatively regulated STAT3-Y705 phosphorylation in normal and malignant keratinocytes. Together, these results underscore that EGFR activation is required but not sufficient for STAT3 activation to occur in malignant keratinocytes. They also highlight complex regulation of STAT3 phosphorylation through EGFR activation including negative regulation via the MAPK kinase/MAPK signaling pathway.

INTRODUCTION

Activation of the epidermal growth factor receptor (EGFR) contributes to multiple aspects of epithelial cell biology relevant to malignant transformation. These include proliferation, migration, and invasion, and modulation of differentiation. Only recently has it become apparent that the EGFR also contributes to survival of epithelial cells including normal and malignant human keratinocytes (1–3). If the EGFR is blocked, normal keratinocytes are significantly more susceptible to induction of apoptotic death by cellular stressors including UVB radiation (4) and matrix detachment (1, 5). Other members of the EGFR family, notably erbB2, serve similar functions relevant to epithelial cell survival (6–10). We have identified one intracellular target of EGFR activation that supports keratinocyte survival (11, 12). Specifically, EGFR activation through endogenous and exogenous ligands is associated with up-regulation of Bcl-x_L, an antiapoptotic member of the Bcl-2 protein family. By contrast, expression of the proapoptotic Bcl-2 family members Bad, Bak, and Bax is not affected by EGFR activation. Thus, EGFR blockade is associated with a proapoptotic bias in the balance of pro- and antiapoptotic Bcl-2 family members. Similar results have since been reported by several other groups (2, 13, 14).

One objective of our previous work was to understand which signaling pathway(s) lead from the EGFR to enhanced cell survival through higher levels of Bcl-x_L expression. These studies demonstrated that EGFR-dependent signals relevant to keratinocyte survival and Bcl-x_L expression are channeled in part through the mitogen-

activated protein kinase (MAPK) kinase (MEK)/MAPK pathway (15). EGFR-dependent MEK/MAPK activation additionally enhances epithelial cell survival by phosphorylation and functional inactivation of the proapoptotic Bcl-2 family member Bad (16). In addition to MEK/MAPK, activation of the signal transducer and activator of transcription (STAT)3 has been implicated in epithelial cell survival and Bcl-x_L expression. STAT3 supports oncogenic transformation of mouse fibroblasts (17, 18) and survival of human squamous carcinoma cells (SCC), at least in part through up-regulation of Bcl-x_L (19). Conversely, EGFR blockade down-regulates STAT3 phosphorylation and DNA binding in some SCC lines consistent with EGFR-dependent regulation of STAT3 activity in this cell system. However, in immortalized keratinocytes, EGFR activation had no apparent effect on STAT3 phosphorylation and, moreover, inhibition of STAT activity by inducible expression of dominant-negative STAT3 constructs had no effect on Bcl-x_L expression in these cells (5).

Collectively, these previous observations raised the issue of whether EGFR-dependent STAT3 activation and its sequelae were restricted to malignant keratinocytes. The present study addresses this question by using normal human keratinocyte cultures, an immortalized keratinocyte line (HaCaT), and a panel of SCC lines exhibiting different levels of EGFR expression and activation states. We demonstrate that: (a) STAT3 phosphorylation on Y705 is a tumor-associated phenomenon in squamous carcinoma cells *in vitro*; (b) STAT3 phosphorylation on Y705 occurs through EGFR-dependent and -independent pathways in malignant cells; (c) overexpression of the EGFR is not sufficient for STAT3 phosphorylation in immortalized keratinocytes (HaCaT); and (d) DNA binding of activated STAT3 is restricted to malignant tumor cells and strictly dependent on EGFR activation. We conclude that STAT3 activation in normal and malignant keratinocytes is a tumor-associated phenomenon linked to deregulated EGFR signaling.

MATERIALS AND METHODS

Reagents and Cells. Properties of the EGFR antagonistic mAb425 have been described earlier (20, 21). Inhibitors to MEK1 (U0126), phosphatidylinositol 3'-kinase (LY294002), SRC-kinases (PP1 and PP2), and the typhostins AG490 and AG1478 were purchased from Calbiochem-Novabiochem (San Diego, CA). Rabbit polyclonal antibodies to EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling/NEB (Beverly, MA), and to β -actin from Amersham Biosciences (Piscataway, NJ). Antibodies to signal transduction components (STAT3, phospho-STAT3, p42/44 MAPK, and phospho-p42/44 MAPK) were from Cell Signaling Technology or Santa Cruz Biotechnology. Hemagglutinin tag antibodies were from Covance (Richmond, CA). Antiphosphotyrosine antibody PY20 was from Transduction Laboratories (San Diego, CA).

Normal foreskin keratinocytes were initiated and maintained in culture as described earlier (22). Head and neck carcinoma cell lines SCC 9 and SCC 12 derived from facial skin were a kind gift from Dr. Jim G. Rheinwald. Head and neck carcinoma cell lines FaDu and Det562 were from the American Type Culture Collection (Rockville, MD). A431 cells were derived from a vulval SCC and originally provided by Dr. I. Pastan. Immortalized keratinocytes (HaCaT cells) were from Dr. Norbert Fusenig (23). HaCaT, SCC 9, SCC 12, FaDu, A431, and Det562 cells were maintained in W489 medium (24) supplemented with 2% FCS. For experiments, all of the cell lines were seeded at

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AQ: B subconfluency in either complete MCDB medium (12) for primary keratinocyte cultures or W489 supplemented with 2% FCS for HaCaT cells and SCC lines. After attachment, medium was replaced with MCDB base medium (12) supplemented with growth factors and inhibitors of signal transduction components as indicated. Inhibitors were diluted from DMSO stocks directly into the culture medium. The DMSO concentration was adjusted to <0.5% in all of the conditions including controls. After 48 h cells were harvested for analysis.

cDNA Constructs and Transfections. A plasmid containing dominant-negative MEK1 (MKK1-8E) was cloned into pCEPT and transfected into HaCaT cells expressing tTA as described previously (15). This system allows transgene induction by removal of tetracycline from culture medium (25). Similarly, dominant-negative STAT3D and STAT3F were cloned into pCEPT and transfected into HaCaT-tTA1 cells as described previously (15). The full-length EGFR cDNA was cloned into the pIRESpuro2 vector (Clontech, Palo Alto, CA) followed by stable transfection into HaCaT cells. Transfections were performed using Eugene 6 (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, cells were seeded at a density of $\sim 5 \times 10^3/\text{cm}^2$ in W 489 medium supplemented with 2% FCS and allowed to attach overnight. The next day transfections were performed using 0.5 μg DNA and 1.7–2.0 μl Eugene 6 per 10^5 cells following the manufacturer's protocol. Selection was started 48–72 h after transfection in W489 medium supplemented with 2% FCS and puromycin at 1 $\mu\text{g}/\text{ml}$ (Sigma-Aldrich, St. Louis, MO).

Immunoprecipitation and Immunoblot Analyses. Samples for immunoprecipitation were collected by washing adherent cells once in ice-cold PBS followed by scraping into cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.0 mM EGTA, 1.0 mM EDTA, 10% glycerol, 1.0% Triton-X-100, 50 mM NaF, 10 mM Na4P2O7 containing freshly added protease inhibitors (Complete protease inhibitor mixture; Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4]. After lysis for 20 min on ice, cell extracts were centrifuged at $15,000 \times g$ and supernatants stored at 70°C until further use. Protein content was determined using the BCA method (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were pre-cleared with 20 μl Protein A/G Plus agarose (Santa Cruz Biotechnology) for 1 h at 4°C . Precleared lysates were then mixed with 2.0 μg of primary rabbit EGFR antibody (Santa Cruz Biotechnology) and 20 μl protein A/G Plus agarose and incubated overnight at 4°C . Precipitates were washed four times with lysis buffer, solubilized in 1 \times reducing Laemmli buffer [62.5 mM Tris-Cl (pH 6.8), 1% SDS, 10% glycerol, 0.5 M 2-mercaptoethanol, or 0.1 MDTT], boiled for 3–5 min, and then subjected to SDS-PAGE followed by Western blot as described below.

AQ: C

Samples for Western blots were collected by washing cells once in cold PBS and lysis in Laemmli buffer followed by boiling for 3–5 min. Equal amounts of protein were separated by SDS-PAGE under reducing conditions and blotted onto nitrocellulose membranes (Millipore). Membranes were blocked using 5% dry milk in PBS or 5% dry milk, 0.05% Tween 20 (Sigma Aldrich) in Tris-buffered saline, and then incubated with primary antibodies in PBS or 5% BSA, 0.05% Tween 20 in Tris-buffered saline, followed by incubation in dilutions of horseradish peroxidase-conjugated secondary antibodies in the same buffers. After antibody incubations, blot membranes were washed in 0.5% Tween 20 in Tris-buffered saline. Signals were visualized by chemiluminescence using reagents from Pierce Chemical Co. according to the manufacturer's instructions. After detection, blots were washed and stripped using Restore Western Blot Stripping Buffer (Pierce Chemical Co.) and used for additional antibody incubations.

Electrophoretic Mobility Shift Assays. Cells were starved for 14 h in growth factor-free medium and then stimulated with epidermal growth factor (EGF) for 15 min. Whole cell lysates were prepared from control and EGF-treated cells. Briefly, after washing twice with PBS, cells were harvested in 1 ml of PBS and recovered by centrifugation. Cells were resuspended in twice the pellet volume using high salt buffer [20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na3VO4, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 20% glycerol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{ml}$ leupeptin] and were kept for 30 min at 4°C under vigorous agitation. The lysates were centrifuged at $15,000 \times g$ for 20 min at 4°C , and protein concentrations of the clarified lysates were determined by Bradford assay. DNA binding was performed by adding 15–20 μg of protein lysates to 18 μl of reaction mixture containing 65 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM DTT, 2% Ficoll 400, 4% glycerol, and 1 μg of poly(deoxyinosinic-deoxycytidylic acid; Amersham Life Science Inc.) The mixture was preincubated on ice

for 15 min followed by addition of 30,000 cpm (1–1.5 ng) of the double-stranded labeled oligonucleotide (high-affinity SIE sequence; Ref. 26) and additionally incubated for 30 min at room temperature. Samples were analyzed in a 20×20 cm 5% polyacrylamide gel with a bisacrylamide:acrylamide ratio of 1:39 containing 2.5% glycerol and $0.5 \times$ TBE (45 mM Tris base and 1 mM EDTA). Electrophoresis was carried out at 175 V until the faster migrating bromophenol blue dye was ~ 4 cm from the bottom edge. The gel was dried and subjected to autoradiography using KodakXAR5 film for 3 days with intensifying screens at -70°C .

RESULTS

Expression and Ligand-Induced Phosphorylation of the EGFR in Normal and Immortalized Keratinocytes and in SCCs. In an earlier study we observed that EGFR activation contributes to STAT3 phosphorylation on Y705 in A431 SCCs but not in immortalized HaCaT keratinocytes (5). These two cell lines were derived from malignant and normal epidermis, respectively. In addition, these cell lines are distinguished by high (A431) and low levels (HaCaT) of EGFR expression. On the basis of these results, we hypothesized that EGFR-dependent STAT3 phosphorylation is restricted to epithelial cells with deregulated EGFR expression and signaling and, hence, a tumor-associated phenomenon. To test this hypothesis we screened a panel of 4 normal primary keratinocytes and 5 malignant SCCs derived either from skin (SCC9, SCC12, and A431) or oral mucosa (all others) for EGFR expression and activation states. For control purposes we included immortalized HaCaT keratinocytes derived from skin (23). We observed that all of the cell lines tested expressed the EGFR albeit at different levels (Fig. 1). When compared with four primary keratinocyte strains and to immortalized HaCaT keratinocytes, the malignant cell lines (Det562, FaDu, SCC12, SCC9, and A431) expressed higher levels of the EGFR with A431 cells expressing the highest. Next, we tested the EGFR phosphorylation state in these cell lines in the presence and absence of exogenous EGF in chemically defined, serum-free media (22). Short-term exposure to 10 ng/ml EGF for 15 min induced EGFR phosphorylation in all of the cell lines as determined by immunoprecipitation of the EGFR followed Western blot analysis using a phosphotyrosine-specific antibody (Fig. 1). The strength of the signal varied with higher levels of EGFR phosphorylation in the transformed cells and the highest level in A431 cells. In the absence of exogenous EGF neither the normal

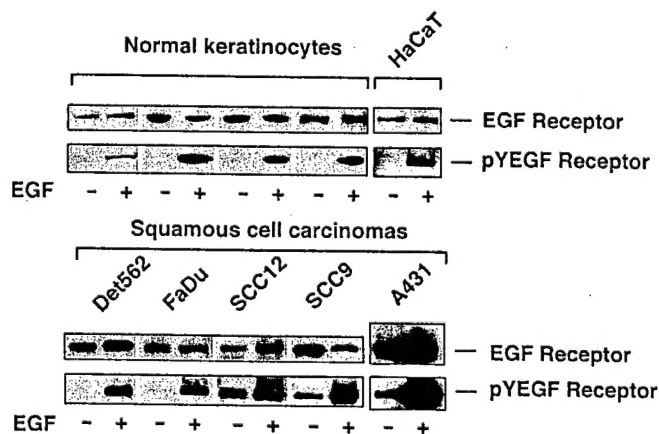


Fig. 1. Expression and phosphorylation of the epidermal growth factor receptor (EGFR) in normal and transformed keratinocytes. Cell extracts were prepared from the cell lines as indicated after stimulation of growth factor-starved cultures with epidermal growth factor (EGF; 10 ng/ml) for 15 min. EGFR expression and phosphorylation levels were determined using antibodies to the extracellular domain of the EGFR and PY20, respectively, by immunoprecipitation and Western blot analyses. For loading controls please refer to Fig. 2, which shows expression levels of STAT3 in the same samples.

keratinocyte strains nor HaCaT cells contained phosphorylated EGFR at detectable levels. In contrast, 3 of 5 malignant cell lines (SCC9, SCC12, and A431) revealed "constitutive" EGFR phosphorylation in the absence of exogenous EGF. In conclusion, the panel of cell lines assembled represents a diverse array of EGFR expression and activation states consistent with deregulated EGFR activation in the malignant cells.

Expression and EGFR-Dependent STAT3 Phosphorylation in Normal and Malignant Keratinocytes. Next, we determined expression and phosphorylation of STAT3 in the panel of cell lines under investigation (Fig. 2). As determined by Western blot analysis all of the cell lines tested expressed STAT3. Consistent with our earlier results (15) addition of EGF to the culture medium induced robust phosphorylation of STAT3 on Y705 in A431 but not in HaCaT cells. STAT3 tyrosine phosphorylation was not detected in any of the 4 normal keratinocyte strains tested either in the presence or absence of exogenous EGF. By contrast, all of the carcinoma cell lines except Det562 revealed STAT3 Y705 phosphorylation in the presence of exogenous EGF. Interestingly, SCC9, SCC12, and FaDu cell extracts contained phosphorylated STAT3-Y705 also in the absence of exogenous EGF. To assess whether the EGFR was functional in the cell lines under investigation we also determined MAPK phosphorylation in all of the conditions. We had observed previously that MAPK phosphorylation in normal keratinocytes and HaCaT cells strictly correlates with EGFR activation under the experimental conditions chosen (15). Consistent with these earlier results, all of the cell lines under investigation here responded to EGF treatment with robust MAPK phosphorylation. The comparatively strong phospho-MAPK signal in some of the keratinocyte extracts is likely due to higher protein concentrations in these samples as evidenced by equally higher levels of STAT3 protein in the same samples.

These results raised the issue of whether STAT3 tyrosine phosphorylation seen in the absence of exogenous EGF in SCC lines was due to EGFR activation via autocrine EGFR ligands. To probe the contribution of endogenous EGFR ligands to STAT3 phosphorylation in FaDu, SCC9, SCC12, and A431 cells we used the EGFR antagonistic tyrphostin AG1478 (Fig. 3A). AG1478 abrogated EGFR phosphorylation (data not shown) and markedly inhibited EGFR-dependent MAPK phosphorylation in all 4 of the SCC lines tested. Yet, AG1478

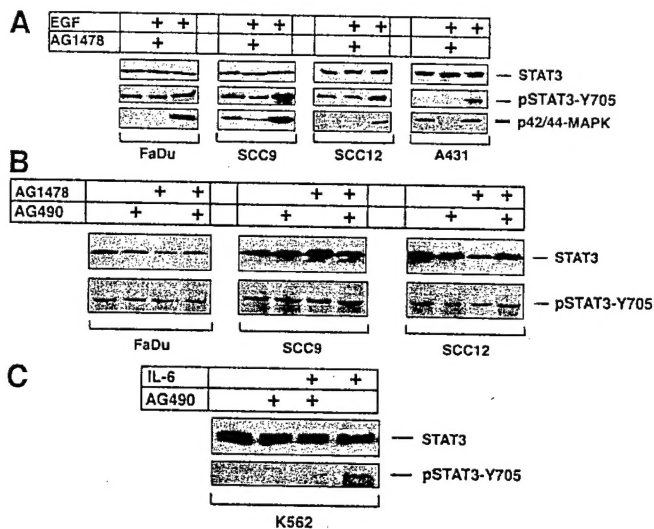


Fig. 3. Effects of inhibitors of the epidermal growth factor (EGF) receptor (AG1478) and of Janus-activated kinase (AG490) alone and in combination on signal transducer and activator of transcription (STAT3) tyrosine phosphorylation in squamous carcinoma cells. Growth factor-starved cells were pretreated with inhibitors for 1 h as indicated and then stimulated with EGF (10 ng/ml) for 15 min before preparing cell extracts. A demonstrates that EGF receptor inhibition by treatment with AG1478 (10 μ M) did not affect STAT3 tyrosine phosphorylation in FaDu, SCC9, and SCC12 in the absence of exogenous EGF. B shows effects of the Janus-activated kinase inhibitor AG490 at 100 μ M on STAT3-Y705 phosphorylation in the absence of exogenous EGF in these cell lines. C documents that AG490 (100 μ M) inhibited interleukin (IL) 6-dependent STAT3-Y705 phosphorylation in K562 cells.

treatment had only marginal effects on STAT3 phosphorylation in those 3 cell lines that demonstrated STAT3-Y705 phosphorylation in the absence of exogenous EGF. Notably, however, AG1478 inhibited the effect of exogenous EGF on STAT3-Y705 phosphorylation in SCC9 and SCC12 cells, and abrogated STAT3 phosphorylation altogether in A431 cells. To ascertain a potential role of gp130 activation in EGFR-independent STAT3 phosphorylation we used the Janus-activated kinase (JAK) inhibitor AG490 (Fig. 3B). AG490 had marginal effects on STAT3 phosphorylation in 2 of the 3 cell lines exhibiting EGFR-independent STAT3-Y705 phosphorylation, although it inhibited interleukin 6-induced STAT3 phosphorylation in K562 erythroleukemia cells (Fig. 3C). A combination of AG1478 and AG490 did not reveal additive effects on EGFR-independent STAT3-Y705 phosphorylation. Furthermore, a triple combination of AG1478/AG490 and SRC kinase inhibitors (PPI or PP2) did not significantly affect EGFR-independent steady-state STAT3 phosphorylation (data not shown). Taken together, these results point to the existence of EGFR-dependent and EGFR/JAK-independent pathways that cooperatively induce STAT3 tyrosine phosphorylation in SCCs.

DNA Binding of STAT3 in Normal and Malignant Keratinocytes. To additionally define the effect of EGFR activation on STAT3 activity in SCCs we performed electrophoretic mobility shift assays using oligonucleotides containing a STAT3 DNA binding motif (Fig. 4). These experiments were done using cell extracts prepared in the presence and absence of exogenous EGF and the EGFR inhibitor AG1478. Only malignant tumor cell extracts produced STAT3:DNA complexes, and this phenomenon was restricted to extracts from EGF-treated cultures. SCC12 cells were a notable exception, because no DNA binding activity was observed either in the presence or absence of exogenous EGF, although these cells showed STAT3-Y705 phosphorylation under these conditions (Fig. 2). As expected, STAT3:DNA complexes were absent in both HaCaT cells and normal keratinocytes regardless of treatment with EGF. Interestingly, tumor cells exhibiting EGFR-independent STAT3-Y705 phosphorylation

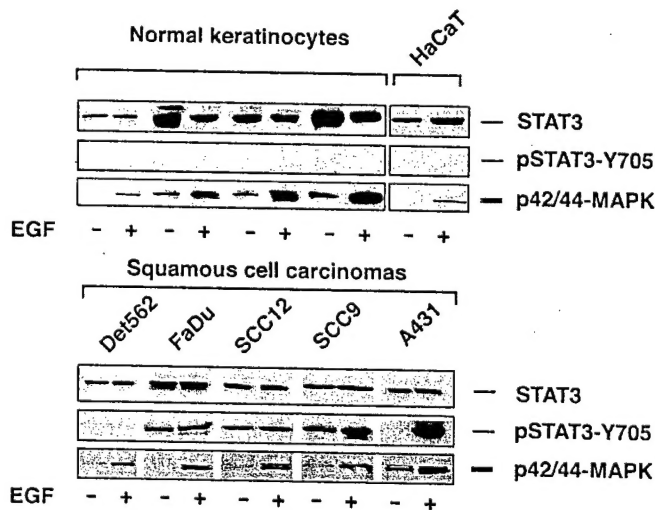


Fig. 2. Expression and phosphorylation states of signal transducer and activator of transcription (STAT3) in normal and transformed keratinocytes. Cell extracts were prepared as described in the legend to Fig. 1 and immunoblots probed using antibodies to STAT3, STAT3 phosphorylated on Y705, and phosphorylated mitogen-activated protein kinase (MAPK) as indicated.

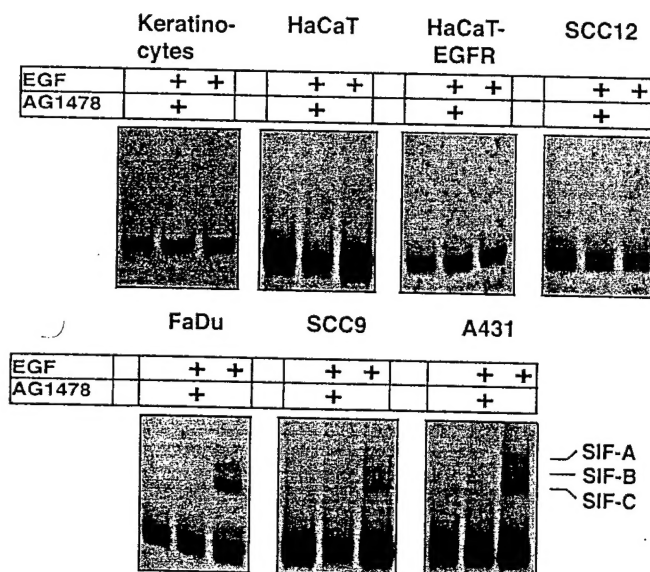


Fig. 4. DNA binding of signal transducer and activator of transcription (STAT3) in SCC lines. Cell lines were treated with epidermal growth factor (EGF; 10 ng/ml) in the presence and absence of AG1478 (10 μ M) as indicated. Cell extracts were prepared and subjected to electrophoretic mobility shift assays with 32 P-labeled STAT3-specific probes. The position of protein/DNA complexes is indicated. The nomenclature of the complexes (SIF-A, -B, and -C) refers to the designations given previously (26). DNA binding activity of STAT3 was restricted to EGF-treated FaDu, SCC9, and A431 cultures.

did not exhibit DNA binding in the absence of exogenous EGF. This result is consistent with the notion that, in malignant keratinocytes, STAT3 phosphorylation on Y705 is not sufficient for transactivation potential.

Absence of STAT3 Phosphorylation in HaCaT Cells Overexpressing the EGFR. Next, we asked the question of whether overexpressing the EGFR in HaCaT cells affects STAT3 tyrosine phosphorylation. To this end, we generated HaCaT cells with levels of EGFR expression similar to those observed in SCCs (HaCaT-EGFR; Fig. 5). Although EGFR phosphorylation in these cells was markedly higher than in parental HaCaT cells and comparable with those observed in SCC9 or SCC12 cells (Fig. 1), neither STAT3-Y705 phosphorylation nor DNA binding activity (Fig. 4) was observed in these cells either in the presence or absence of exogenous EGF.

Inhibition of STAT3 Phosphorylation in Normal and Malignant Keratinocytes by EGFR-Dependent MEK Activity. This result raised the issue of whether, in HaCaT keratinocytes, STAT3 tyrosine phosphorylation can be induced by any means. To this end, we first used HaCaT cells conditionally overexpressing a dominant-negative but phosphorylatable STAT3 construct (STAT3D) as described earlier (15, 27). As shown in Fig. 6A, upon overexpression of STAT3D in HaCaT cells, robust STAT3-Y705 phosphorylation was observed. By contrast and as expected, conditional overexpression of a dominant-negative construct in which Y705 had been replaced by phenylalanine (STAT3F; Ref. 27) did not result in STAT3-Y705 phosphorylation under these conditions. Next, we assessed whether STAT3 expressed at physiological levels could be phosphorylated on Y705 by using the MEK inhibitor U0126 (Fig. 6B). This was done based on previous reports, which demonstrated that blocking MEK enhances interleukin 6-dependent STAT3 tyrosine phosphorylation in HepG2 hepatoma and MM6 myeloma cells (28, 29). Consistent with these earlier reports pharmacological inhibition of MEK activity with the U0126 compound was accompanied not only by reduced phosphorylation of MAPK but also by increased STAT3 Y705 phosphorylation in HaCaT cells. Interestingly, this effect was obvious only in the presence of exogenous EGF. Similarly, blocking MEK activity in SCC9, FaDu,

SCC12, and A431 cells with U0126 was associated with enhanced levels of STAT3 Y705 phosphorylation in response to exogenous EGF. To ascertain that this effect was due to inhibition of MEK rather than nonspecific effects of U0126, we used HaCaT keratinocytes engineered to express dominant-negative MEK in an inducible fashion as described previously (15). Induction of the transgene in these

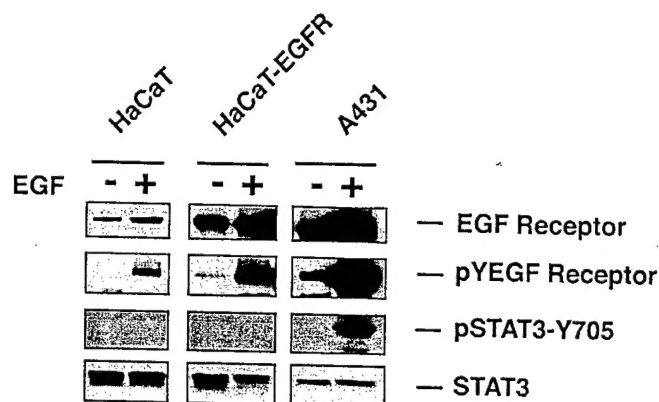


Fig. 5. Effect of forced expression of the epidermal growth factor (EGF) receptor on signal transducer and activator of transcription (STAT3) phosphorylation in HaCaT cells. Expression and autophosphorylation states of the EGF receptor were assessed in HaCaT cells, HaCaT cells engineered to express high levels of the EGF receptor, and A431 cells as controls. In all cases, cells were treated with EGF for 15 min before preparing cell extracts and Western blot analyses with antibodies as indicated.

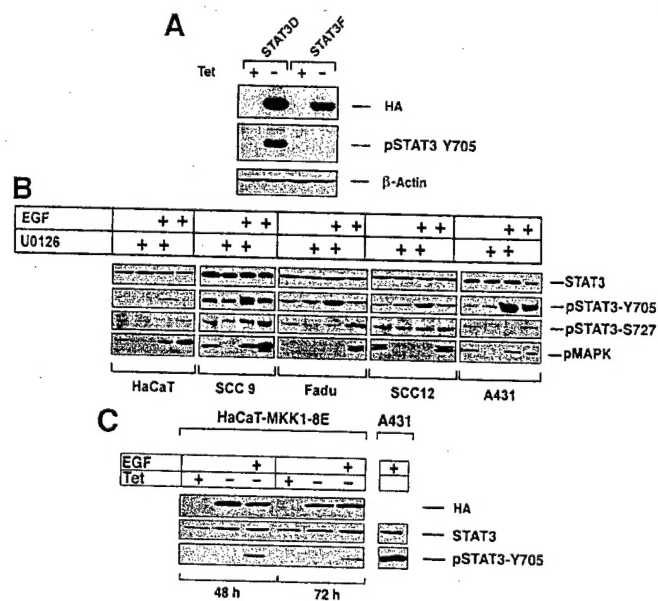


Fig. 6. Modifiers of signal transducer and activator of transcription (STAT3) tyrosine phosphorylation in immortalized and malignant epithelial cells. A demonstrates that induced expression of a phosphorylatable STAT3 construct (STAT3D) in HaCaT keratinocytes is associated with constitutive tyrosine phosphorylation of this construct. Induction of the transgene by removal of tetracycline (Tet) from the culture medium was detected by Western blot analysis using an antibody against the hemagglutinin tag. Phosphorylation of STAT3 was probed using an antibody that specifically recognizes STAT3-Y705. As a loading control the expression of β -actin was determined in the same samples. A nonphosphorylatable STAT3 construct included as a negative control (STAT3F) was not phosphorylated. B shows induction of epidermal growth factor (EGF) receptor-dependent STAT3-Y705 phosphorylation in immortalized HaCaT cells and in squamous cell carcinoma (SCC) cells by treatment of cells with the mitogen-activated protein kinase kinase inhibitor U0126. C demonstrates induction of EGF receptor-dependent STAT3-Y705 phosphorylation in HaCaT cells expressing a dominant-negative MEK1 construct (MKK1-8E). STAT3 phosphorylation was detected upon induction of the hemagglutinin (HA)-tagged transgene by removal of tetracycline from the culture medium and was strictly dependent upon the presence of exogenous EGF. Expression and tyrosine phosphorylation of STAT3 in control A431 cells are also shown.

cells also led to STAT3-Y705 phosphorylation in a strictly EGFR-dependent manner (Fig. 6C). These results demonstrate that EGFR activation can elicit STAT3 phosphorylation on Y705 in HaCaT cells. However, for this effect to take place concomitant MEK activation needs to be inhibited.

EGFR-Dependent STAT3 Serine Phosphorylation. It has been described previously that EGFR activation leads to phosphorylation of STAT3 on S727 through a MAPK-dependent pathway (28). Therefore, we assessed patterns of dual phosphorylation of STAT3 on Y705 and S727 in the presence of EGF in SCCs (Fig. 6B). Because S727 phosphorylation appears to be induced by MAPK in other cell systems we also tested the effect of the MEK inhibitor U0126 on S727 STAT3 phosphorylation. The results of this analysis showed S727 phosphorylation in all of the cell lines in the absence of exogenous EGF. Addition of EGF led to increased S727 phosphorylation in SCC9 and FaDu cells but not in HaCaT, SCC12, or A431 cells. Treatment with U0126 moderately reduced but did not abrogate S727 phosphorylation in HaCaT, SCC9, FaDu, and A431 cells in the presence and absence of exogenous EGF. By contrast, only marginal effects of U0126 were observed in SCC12 cells. These results suggest that STAT3-S727 phosphorylation in SCC lines is, in part, dependent on MEK activity. Yet, no obvious relationship between EGF-dependent STAT3-S727 phosphorylation and DNA binding of STAT3 emerged in the panel of cell lines investigated here. Specifically, in A431 cells, EGF induced robust DNA binding, yet STAT3-S727 phosphorylation was not affected. In addition, comparatively high levels of STAT3-S727 phosphorylation were observed in SCC12 cells, which revealed no STAT3/DNA binding activity either in the absence or presence of exogenous EGF. We conclude that EGFR phosphorylation is likely to contribute a signal distinct from Y705 and S727 phosphorylation that is necessary to acquire STAT3 DNA binding competence.

DISCUSSION

The present study was designed to test the hypothesis that STAT3 activation is a tumor-associated phenomenon associated with deregulated EGFR signaling in SCCs. Whereas the results obtained strongly support this contention they also raise several unexpected issues about STAT3 activation in normal and malignant keratinocytes. First and foremost we observed that dual STAT3 phosphorylation on Y705 and S727 was not sufficient to induce STAT3 DNA binding in this cell type. These results are reminiscent of a recent study by Bild *et al.* (30) who demonstrated that, in addition to phosphorylation, endocytotic transport of STAT3, presumably complexed with the activated EGFR, is a necessary prerequisite for nuclear import, DNA binding, and transcriptional activity in NIH3T3 cells engineered to overexpress the EGFR.

Another rather unexpected result of this study related to the presence of pSTAT3-Y705 in growth factor-starved malignant epithelial cells. Furthermore, STAT3-Y705 phosphorylation persisted in these cells in the presence of the EGFR inhibitor AG1478. During preparation of this article a similar observation was reported using a different set of head and neck SCCs (31). In that report EGFR-independent STAT3-Y705 phosphorylation was ascribed to autocrine interleukin 6/JAK-dependent signaling. Our results provide only limited support for this conclusion, because treatment with the JAK inhibitor AG490 reduced pSTAT3-Y705 content in only one (SCC12) of the three cell lines tested. By contrast, AG490 had no detectable effect on pSTAT3-Y705 content in SCC9 and FaDu cells. Similarly, the kinase responsible for STAT3-Y705 phosphorylation in these SCC cells is not a SRC-like kinase, because SRC inhibitors (PPI/PP2) had only negligible effects on EGFR-independent STAT3-Y705 phos-

phorylation.³ Thus, we have excluded the three major pathways known to contribute to STAT3 tyrosine phosphorylation, *i.e.*, the EGFR, a SRC-like kinase, and gp130/JAK in the maintenance of STAT3 phosphorylation in the growth factor-starved state of SCC in cell culture. Identification of the kinase responsible for this constitutive STAT3-Y705 phosphorylation awaits future studies.

A third observation of interest relates to the finding that EGFR-dependent activation of MEK/MAPK signaling negatively regulates STAT3-Y705 phosphorylation in HaCaT cells and in SCCs. Although this phenomenon has been described before in another cell type (28), the involvement of MEK in those systems was implied only by using pharmacological MEK inhibitors, which are known to also inhibit other members of the MEK family, for example, MEK5 (32). By using HaCaT cells conditionally overexpressing dominant-negative MEK we were able to provide independent evidence confirming that EGFR-dependent MEK activation serves to suppress EGFR-dependent STAT3-Y705 phosphorylation in HaCaT cells. Moreover, we used cells expressing physiological levels of STAT3 to make this observation. This experimental detail is of considerable importance, because we observed that forced overexpression of STAT3 led to robust Y705 phosphorylation irrespective of regulatory influences (see Fig. 6A).

In conclusion, the results of the present study highlight complex regulation of STAT3 activation in SCC lines. Of largest practical import is our finding that STAT3 activation is a tumor-associated phenomenon in this cell system and strictly dependent on EGFR activation. Furthermore, our results illuminate the importance of distinguishing STAT3 phosphorylation events from DNA binding activities. Although STAT3 tyrosine phosphorylation appears to be necessary for biological activity, only DNA binding and subsequent transcriptional effects are likely to contribute to STAT3 function relevant to the malignant phenotype.

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